



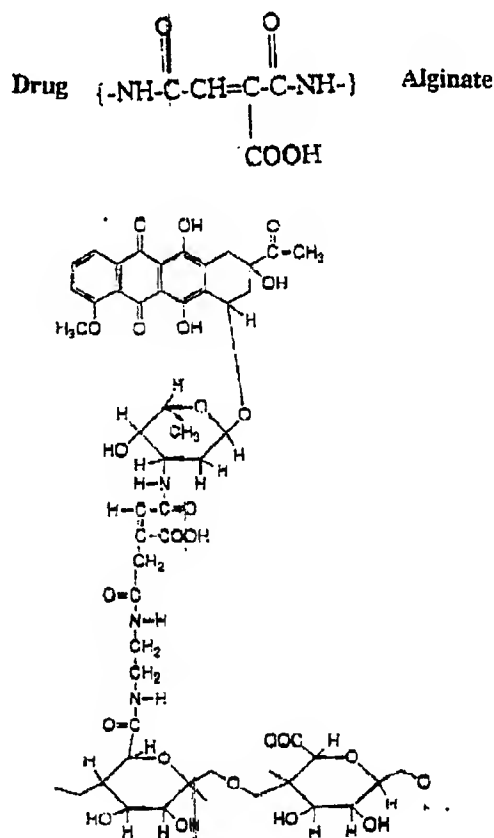
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 47/48, 51/71	A2	(11) International Publication Number: WO 94/07536	
		(43) International Publication Date: 14 April 1994 (14.04.94)	
(21) International Application Number: PCT/GB93/01999		(74) Agents: SKERRETT, H., N. et al.; Charles House, 148/9 Great Charles Street, Birmingham B3 3HT (GB).	
(22) International Filing Date: 23 September 1993 (23.09.93)			
(30) Priority data: 9220294.4 25 September 1992 (25.09.92) GB		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(71) Applicant (for all designated States except US): KEELE UNIVERSITY [GB/GB]; Staffordshire ST5 5BG (GB).		Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(72) Inventors; and			
(75) Inventors/Applicants (for US only) : AL-SHAMKHANI, Ay-men [IQ/GB]; Sir William Dunn School of Pathology, Oxford University, South Parks Road, Oxford OX1 3RE (GB). DUNCAN, Ruth [GB/IT]; Farmitalia Caro Erba, Via Carlo Imbinati, 24, I-20159 Milano (IT).			

(54) Title: ALGINATE-BIOACTIVE AGENT CONJUGATES

(57) Abstract

The invention is an alginate-bioactive agent conjugate connected via an acid labile biodegradable spacer linkage. The conjugate is effective for delivering bioactive agents to targets existing in low-pH environments, either at the target surface or in the target interior.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

TITLE OF THE INVENTION

ALGINATE-BIOACTIVE AGENT CONJUGATES

5 BACKGROUND OF THE INVENTION

One of the significant drawbacks of antitumor or antineoplastic agents is their failure to discriminate between normal dividing cells and tumor cells. When acting on normal dividing cells, these agents cause undesirable side effects, including nausea, apolecia, and bone marrow toxicity. Their cardiotoxicity is a primary contributor to dosage limitation.

10 Kopecek et al., United States Patent 5,037,883, describe a drug conjugate which includes inert synthetic N-(2 hydroxypropyl) methacrylamide polymeric carriers combined through peptide spacers with a bioactive molecule, with a targeting moiety, and with an optional cross-linkage. The peptide spacers contain between 2 and 6 naturally occurring amino acids.

15 Yang et al., *Proc. Natl. Acad. Sci. USA* vol. 85, pp. 1189-1193 (1988) describe doxorubicin conjugated with a monoclonal antibody directed to a human melanoma-associated proteoglycan. The conjugate suppresses the growth of established tumor xenografts in nude mice.

20 Dillman et al., *Cancer Research* vol. 48, pp. 6097-6102 (1988) describe the superiority of an acid-labile daunorubicin-monoclonal antibody immunoconjugate compared to free acid.

25 Shen et al., *Biochemical and Biophysical Research Communications* vol. 102, no. 3, pp. 1048-1054 (1981) describe cis-aconityl spacer between daunomycin and macromolecular carriers.

30

SUMMARY OF THE INVENTION

The invention is an alginate-bioactive agent conjugate which comprises:

- 5 (a) an alginate or alginate derivative; and
- (b) a bioactive agent,

10 wherein the alginate and the bioactive agent are connected via a biodegradable spacer linkage. Preferably, the linkage is an acid labile linkage.

The present invention also comprises pharmaceutical compositions which comprise at least one conjugate of the invention and an inert, physiologically acceptable carrier. The
15 compositions can be administered orally or by injection, for example by intraperitoneal, intravenous or intramuscular injection.

20 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows percent daunomycin *in vitro* release over time.

25 Figure 2 shows $\mu\text{g/ml}$ daunomycin *in vitro* release over time.

Figure 3 shows the effect of daunomycin release on tumor cell growth.

30

DETAILED DESCRIPTION OF THE INVENTION

The invention permits combination of controlled release of a bioactive material and the beneficial biological properties of alginates. Non-toxic alginates display inherent properties which
5 provoke beneficial pharmacological activity and when covalently bound to a bioactive material (including drugs and prodrugs), alginates can be used to control the rate of release of the material.

Alginate, as used herein, refers to sodium alginate, alginic acid sodium salt or algin (see page 41, The Merck Index,
10 11th Edition (1989), Merck & Co., Inc.) Alginates are gelling polysaccharides extracted from giant brown seaweed (Macrocystis pyrifera) or horsetail kelp (Laminaria digitata) or from sugar kelp (Laminaria saccharina). In seaweed, polysaccharides are present as a mixed sodium and/or potassium, calcium and magnesium salt.
15 Alginates are commercially available from Kelco, a Division of Merck & Co., Inc. (San Diego, CA).

Various alginates useful in this invention are described in detail by I.W. Cottrell and P. Kovacs in "Alginates", Chapter 2 of Davidson, ed., Handbook of Water-Soluble Gums and Resins (1980).
20 Most preferred herein are naturally derived algal sodium alginates (also called "algin" or "alginic acid sodium salt"), such as those sold commercially under the trademarks KELTEX®, KELGIN® and KELTONE™ by Kelco Division of Merck & Co., Inc.

Alginates also include "bioalgin", microbially produced
25 polysaccharides produced by both Pseudomonas and Azotobacter strains as described, for example, in Jarman et al., United States Patent 4,235,966. These alginates are polysaccharides consisting of a partially acetylated variable block copolymer of D-mannuronic and L-guluronic acid residues. Jarman et al. state that the polysaccharide produced is
30 similar to that produced from seaweed except that the molecule is partially acetylated.

Alginate contains both mannuronic acid and guluronic acid. The presence of three kinds of polymer segments in alginic acid from various brown algae has been shown. One segment consists essentially of D-mannuronic acids units; a second segment consists essentially of L-guluronic acid units; and the third segment consists of alternating D-mannuronic acid and L-guluronic acid residues.

The proportions of the three polymer segments in alginic acid samples from different sources have been determined using partial acid hydrolysis to separate the alginic acid into homopolymeric and alternating segments.

One of the most important and useful properties of alginates is the ability to form gels by reaction with calcium salts. These gels, which resemble a solid in retaining their shape and resisting stress, consist of almost 100% water (normally, 99.0 to 99.5% water and 0.5 to 1.0% alginate).

A gel, in classical colloid terminology, is defined as a system which owes its characteristic properties to a cross-linked network of polymer chains which form at the gel point.

Alginates have the ability to stimulate the production of cytokines such as tumor necrosis factor-alpha, interleukin-6, and interleukin-1. In one embodiment of the invention, alginates are conjugated to antitumor agents. Conjugation of the antitumor agent to alginate effectively improves the therapeutic index of the drug by controlling release while the alginate carrier stimulates an antitumor cellular immune response.

In another embodiment of the invention, alginates are conjugated to antibiotics. These conjugated antibiotics are especially useful for treating superficial wounds. The conjugate effectively controls the release of the antibiotic at the site of injury, and the alginates promote wound healing.

The invention is an alginate-bioactive agent conjugate which comprises:

(a) an alginate or alginate derivative; and

(b) a bioactive agent,

wherein the alginate and the bioactive agent are covalently bound via a biodegradable linkage such as an acid labile linkage.

The conjugates of the invention may be administered to organisms, including mammals, preferably in combination with pharmaceutically acceptable carriers or diluents, optionally with known adjuvants such as alum, in a pharmaceutical composition which is non-toxic and in a therapeutically effective amount, according to standard pharmaceutical practice. The compounds can be administered orally, parenterally (e.g. by intravenous, subcutaneous, intramuscular, or intramedullary injection), topically, or percutaneously (e.g. by diadermic, transcutaneous, or transdermic procedures).

For oral use, the conjugates may be administered, for example, in the form of tablets or capsules, or as an aqueous solution or suspension. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also commonly used. For oral administration in capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents may be added.

Alginate-bioactive agent conjugates can be mixed, for example, with alginate, and subsequently gelled by the addition of CaCl_2 to form alginate beads. Alginates behave differently at varying pH

levels (between 2 and 7) which allows for further control of drug release.

For intramuscular, intraperitoneal, subcutaneous and intravenous use, sterile solutions of the active ingredient are usually prepared, and the pH of the solutions should be suitably adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled in order to render the preparation isotonic.

Preferably, the alginate comprises a first segment consisting essentially of D-mannuronic acid residues, a second segment consisting essentially of L-guluronic acid residues, and a third segment consisting essentially of alternating D-mannuronic acid and L-guluronic acid residues.

The molecular weight of the alginate must be large enough to prevent rapid passage through the kidney of the patient and yet small enough to assure passage within a reasonable time. The alginate molecular weight is preferably between about 2,000 and 1,000,000. More preferred molecular weights depend on the method of administration of the bioactive material. For bioactive materials which are administered parenterally, alginates having molecular weights of between about 5,000 and 100,000 are preferred. For bioactive materials which are administered orally, such as in a capsule or tablet, higher molecular weight alginates, between 200,000 and 1,000,000, are preferred.

The alginate preferably has attached amino groups to which the conjugate linkage is attached.

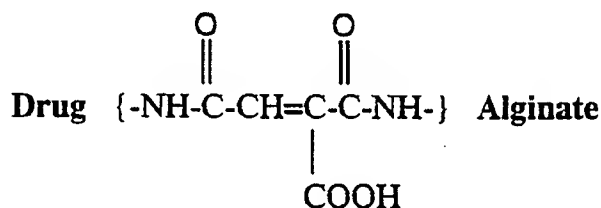
The preferred bioactive agent is selected from the group of antitumor agents and antibiotics. The preferred antitumor agent is a cytotoxic drug such as an anthracycline or derivative thereof. The anthracycline is preferably daunorubicin (also referred to as daunomycin or Dnm), doxorubicin, epirubicin or

derivatives thereof, more preferably, daunorubicin. The preferred antibiotic is amoxycillin or ampicillin.

Other suitable bioactive materials are those having functional NH_2 groups for binding the alginate, such as bioactive proteins or peptides, or other therapeutic compounds which are first modified to include the appropriate functional group, e.g. 5-aminosalicylic acid, prior to conjugation with the alginate.

The acid labile linkage is physiologically stable at vascular pH of about 7.4 and hydrolytically cleaves under acidic condition. Release of the drug to which the alginate is conjugated occurs in any acidic environment, such as a mammal's gastrointestinal tract, at the site of inflammation, inside a tumor mass, or intracellularly.

Preferably, the acid labile linkage between the drug and alginate is a cis-aconityl group having the formula



wherein the group is attached to drug via peptide bond and attached to alginate via peptide bond.

Preferably, when the bioactive material is a cytotoxic drug, the conjugates of the present invention preserve cytotoxic pharmacological activity and allow pH dependent release. The conjugate releases the cytotoxic agent in the acidic milieu of the endosome/lysosome and the acidic environment or surroundings of the tumor. The toxicity of the cytotoxic agent is reduced as the complex is not hydrolyzed in the plasma at neutral pH.

The invention is also a method for releasing a cytotoxic drug in an acidic environment comprising

- 5 (a) introducing an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and the cytotoxic drug are connected via an acid labile linkage, to a pH environment of about 7.4;
- 10 (b) modifying the pH environment of the conjugate by decreasing the pH; and
- (c) hydrolyzing the alginate-cytotoxic drug conjugate linkage.

15 The invention is also a method for releasing a cytotoxic drug in an acidic environment comprising

- 20 (a) introducing an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and the cytotoxic drug are connected via an acid labile linkage, into an acidic body compartment, such as a stomach or tumor cell; and
- 25 (b) hydrolyzing the alginate-cytotoxic drug conjugate linkage.

The invention is also a method for delivering a cytotoxic drug to a tumor which comprises

- 30 (a) introducing to the tumor cell surface an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and

the cytotoxic drug are connected via an acid labile linkage;
and,

5 (b) hydrolyzing the alginate-cytotoxic drug conjugate linkage
in the low pH surroundings of the tumor surface to release the
cytotoxic drug to the tumor surroundings and absorbing the
cytotoxic drug through the cellular membrane.

10 The invention is also a method for treating a neoplastic
condition in a patient having a tumor which comprises

(a) introducing an alginate-cytotoxic drug conjugate
comprising (i) an alginate or alginate derivative, and (ii) a
15 cytotoxic drug, wherein the alginate and the cytotoxic drug
are connected via an acid labile linkage, to a patient having a
neoplastic condition; and

(b) hydrolyzing the alginate-cytotoxic drug conjugate linkage
20 in the low pH surroundings of the tumor surface to release the
cytotoxic drug to the tumor surroundings and absorbing the
cytotoxic drug through the cellular membrane.

25 The invention is also a method for delivering a cytotoxic
drug to a tumor which comprises

(a) introducing an alginate-cytotoxic drug conjugate
comprising (i) an alginate or alginate derivative, and (ii) a
cytotoxic drug, wherein the alginate and the cytotoxic drug are
30 connected via an acid labile linkage, to the tumor cell surface;

(b) internalizing the conjugate by endocytosis into the tumor;
and,

(c) hydrolyzing the alginate-cytotoxic drug conjugate linkage in the low pH environment of the tumor interior to release the cytotoxic drug inside the tumor.

5

The invention is also a method for treating a neoplastic condition in a patient having a tumor which comprises

10

(a) introducing an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and the cytotoxic drug are connected via an acid labile linkage, to the tumor cell surface;

15

(b) internalizing the conjugate by endocytosis into the tumor; and,

20

(c) hydrolyzing the alginate-cytotoxic drug conjugate linkage in the low pH environment of the tumor interior to release the cytotoxin inside the tumor.

The general strategy for preparing the conjugate

Alginate - S' - B'

25

wherein S' is a pH sensitive biodegradable linker and B' is a bioactive agent, is as follows:

a) $S' + B'-NH_2 \longrightarrow HOOC-S'-B'$

30

b) $Alginate \xrightarrow{EDC} "Alginate"$

c) "Alginate" + ethylenediamine -----> Alginate-NH₂

d) $\text{HOOC-S'-B'} \xrightarrow{\text{EDC}} \text{"S'-B"}$

e) Alginate-NH₂ + "S'-B" -----> Alginate-S'-B'

"Alginate" is activated alginate, and "S'-B" is activated S'-B'.
EDC is 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

Generally, the procedure for preparing alginate-bioactive agent conjugates, preferably alginate-cytotoxic drug conjugates, involves the steps of:

a) combining the linker with the cytotoxic drug to form a linker-modified cytotoxic drug;

b) activating the alginate with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide;

c) combining activated alginate with ethylenediamine to form alginates having free primary amine groups (alginate-NH₂);

d) activating the linker-modified cytotoxic drug with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; and

e) combining activated linker-modified cytotoxic drug with alginate-NH₂ to form the alginate-cytotoxic drug conjugate.

Alginates

Alginates of various molecular weights are commercially available from Kelco. Molecular weights can also be modified according to the following procedure.

5 Preparation of low molecular weight Alginates

10 A method to obtain alginates with a molecular weight of ~100 kDa is described below. Low molecular weight alginates (identified below as F1, F2, and F3, were produced by controlled degradation of alginate(56) (alginate having a molecular weight of about 427 kDa) as follows: samples of alginate(56) dissolved in acetic acid (1% v/v) were subjected to the following conditions:

- 15 (a) Heating to 121°C for 15 minutes, or
 (b) Heating to 105°C for 5 minutes, or
 (c) Heating to 100°C for 5 minutes.

20 The resulting solutions were neutralized and then dialyzed extensively against distilled water, followed by freeze drying and then analysis by SE-HPLC. The appropriate alginates (~131 kDa) were obtained by heating to 100°C for 5 minutes.

The following table describes various alginates used to prepare conjugates of the present invention.

25 Alginate samples

<u>Reference</u>	<u>M/G</u>	<u>Seaweed species</u>	<u>Mw</u>	<u>Mw/Mn</u>
56	high	<i>Macrocystis pyrifera</i>	427000	3.1
55	low	mixture of high "G" seaweeds	763000	2.9
30 58	high	<i>Macrocystis pyrifera</i>	689000	2.7
Asco	high	<i>Ascophyllum nodosum</i>	690000	3.5
Flavi	low	<i>Lessonia flavicans</i>	797000	2.9

-	-	from alginate(56)	131000	2.8
-	-	from alginate(56)	56000	2.9
F3	-	from alginate(56)	10000	1.9

5 M/G is mannuronic acid:gluronic acid ratio. Mw is average molecular weight as determined by size exclusion - HPLC using pullulan standards. Mw/Mn is polydispersity. High "G" seaweeds include *Lessonia flavicans* and *Laminara hyperborea*. Alginate(58) is propylene glycol alginate.

10 Biocompatibility assessment of various alginates by the MTT assay

The assay, used for evaluating polymers and drugs, is based on the ability of viable cells to reduce a soluble tetrazolium dye, MTT, to colored water insoluble formazan crystals. The assay was carried out in 96-well microtitre plates (flat bottomed).

15 At confluence, B16F10 cells were detached as follows: following removal of the medium, cells were washed twice with sterile PBS to remove excess medium. Trypsin-EDTA solution (2ml) was added and the flasks were agitated gently for 30 seconds. The trypsin-EDTA solution was then removed using a
20 Pasteur pipette connected to a vacuum line and the cells incubated at 37°C for 1 minute to allow detachment. Cells were suspended in medium (5ml/flask) and diluted accordingly to give a concentration of 10^4 cells/ml.

25 Medium containing cells (200µl) were dispensed by a multichannel pipette into each well with the exception of the first row wells which were filled with medium only (200µl) and were designated "blank" wells (used for zeroing with the microtitre plate reader). A seeding density of 10^4 cells/ml was previously found optimal to B16F10 cells since polymers/drugs could be
30 added 24 hours after cell plating, just as cells were beginning to enter their exponential phase and the MTT assay performed 72 hours later giving a mean value for the absorbance of formazan of

approximately 1.5 for the untreated controls. Following an incubation of 24 hours at 37°C (5%CO₂), the medium was removed by aspiration and in some wells replaced by medium containing polymers/drugs at various concentration (200µl, 6 replicates). The remaining wells were replaced with medium only (200µl) and designated "untreated controls." Due to the poor solubility of alginates in the growth medium-MEM (due to the presence of high concentration of divalent metals), all alginate stock solutions (10mg/ml) were prepared in Ham's F10 medium, supplemented with foetal calf serum (10%v/v) and L-glutamine (1%v/v). The stock solutions were then sterilized by filtration (Flowpore D, 0.2µm filter) and diluted with MEM prior to addition to cells. Any detrimental effects on the cells due to Ham's F10 medium were accounted for by control wells where Ham's F10 medium was diluted in the same ratio with MEM and then added to cells.

Following a 67 hour incubation (37°C, 5% CO₂), a sterile solution of MTT (20µl, 5mg/ml in PBS) was added to each well, and the plates were placed in the incubator for a further 5 hour period (72 hour in total). At the same time period, the medium was removed with the aid of a hypodermic needle connected to a vacuum line. The formazan crystals were dissolved by the addition of spectrophotometric grade DMSO (200µl) to each well and the absorbance determined at 550nm using a microtitre plate reader.

The results of each treatment were expressed as a percentage of the appropriate controls (untreated cells).

The following alginates were tested against the mouse melanoma cell line B16F10:

5	<u>Alginate</u>	<u>concentration range (mg/ml)</u>
	56	0.1 - 5.0
	55	0.1 - 5.0
	58	0.1 - 5.0
	Asco	0.1 - 2.5*
10	Flavi	0.1 - 1.0*
	Alginate 427 kDa	0.05 - 1.0
	Alginate 90kDa	0.05 - 1.0
	Alginate 35kDa	0.05 - 1.0
15	F3	0.05 - 1.0

* higher concentrations could not be used as they were too viscous to be filtered through 0.2 m filters.

All the alginates tested were not toxic at the concentrations used in these experiments, with the exception of propyleneglycol alginate (PGA) which was toxic at 5 mg/ml. As expected, poly-L-lysine was toxic at even very low concentrations. The toxicity of PGA may be due to covalent coupling of cell surface membrane proteins due to the reaction of the primary amine groups of proteins with the reactive ester groups of the polymer. This could lead to membrane perturbation and eventually cell death. Toxicity may have also resulted from propyleneglycol which is likely to be released from the alginates upon incubation in tissue culture.

30 Example 1

Preparation of alginate-NH₂

Alginate(56) (500 mg) was dissolved in water (50 ml) and pH adjusted to 4.75 with HCl (0.1 M). Solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (60 mg), was added and pH kept constant by addition of HCl (0.1 M). After 1h, the solution was added dropwise to a solution containing a 40-fold excess of ethylene diamine. The pH was adjusted to 4.75 with NaOH (0.1 M) and the solution left stirring at room temperature. One hour later, the pH was raised to 6 and the mixture left stirring for another hour. The pH was then increased to 12 with NaOH (5 M) and NaCl (4.5 g) was added. This was necessary in order to dissociate electrostatically bound diamine. The final solution was then extensively dialysed, firstly against carbonate buffer (pH 11.3) and then against distilled water.

A control was also carried out where alginate was mixed with ethylene diamine without prior activation with EDC. Using an identical protocol, alginate-tyrosinamide containing amine groups was produced.

The amine content of the polymers was estimated by the o-phthalaldehyde method.

Preparation of biactive agents for conjugation to alginate-NH₂
N-cis-aconityl-Duanomycin (N-cis-aconityl-Dnm)

Dnm (6 mg) was dissolved in 1 ml Na₂HPO₄ buffer (pH 9.0) and kept on ice. cis-Aconitic anhydride (10 mg) was then added slowly while stirring, and the pH was kept at 9 by the addition of NaOH (0.5 M). The solution was mixed at 0°C for 10 minutes and then at room temperature for 15 minutes. The reaction mixture was then made up to 3 ml with water and left on ice.

N-cis-aconityl-Dnm was precipitated with HCl (1.0 M) and kept on ice for 0.5 hours. After centrifugation, the precipitate was dissolved in water with small additions of NaOH (0.1 M).

The purification procedure was repeated and the final precipitate was dissolved in water (0.5 ml) with small additions of NaOH (0.1 M). The concentration of the product was determined by measuring the absorbance at 475 nm. The product was checked by
5 TLC (solvent: dichloromethane/methanol/acetic acid/water - 30:4:1:0.5).

Synthesis of Alginate-Daunomycin Conjugates

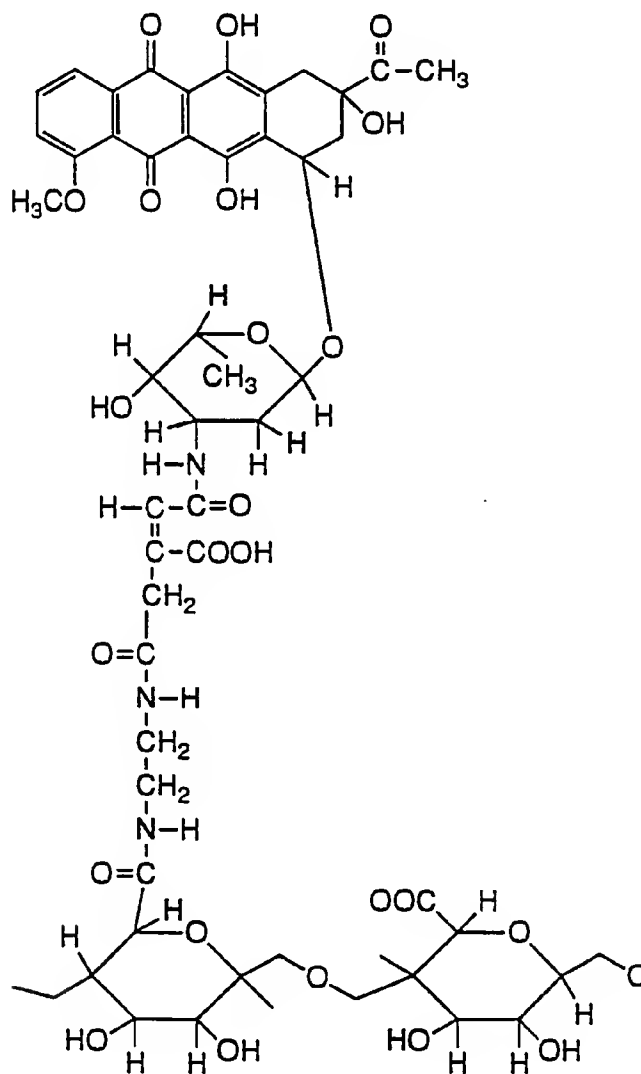
The anthracycline antibiotics are the most widely used antitumor agents. Daunomycin is mainly used in the treatment of
10 leukaemias, but as in the case of all antineoplastic agents, daunomycin does not discriminate well between normal dividing cells and tumor cells and is associated with side-effects such as nausea, alopecia and bone marrow toxicity. In addition daunomycin shows cardiotoxicity which is often dose limiting.

15 Alginate-Dnm conjugates provide advantages over and above the existing conjugates. First, daunomycin is coupled to alginate via an acid-sensitive linker, cis-aconitic anhydride, which is stable at the vascular pH of 7.4. Once the macromolecular conjugate is endocytosed by the target cell, the drug is cleaved in
20 the more acidic environment of the endosomes and lysosomes via a mechanism of hydrolytic cleavage. Second, the alginate carrier is biodegradable *in vivo*, and the carrier is therefore more easily removed from the organism.

25 Two types of alginates were used for this synthesis: high 'M' alginate(56) and alginate-tyrosinamide. The latter was used in order to radiolabel the conjugate with ^{125}I to enable us to study its pharmacokinetics.

30 To a solution of N-cis-aconityl-Dnm (pH 7.0) an equivalent amount of solid EDC was added. The solution was stirred for 20 minutes at room temperature and then alginate-NH₂ solution (1% w/v) was added dropwise with continuous stirring. The mixture was left reacting for 5 hours (Alginate-tyrosinamide-NH₂) or 24 hours (alginate(56)-NH₂).

Free daunomycin was separated from polymer bound on Sephadex G50 column (2.6 x 60 cm). The amount of bound daunomycin was determined by measuring the absorbance at 475 nm, and free daunomycin was found to be absent using TLC. The product structure is shown below:



Structure of Alginate-Daunomycin conjugate

Alginates containing free primary amine groups were successfully synthesized. The amount of ionically bound diamine was almost negligible.

<u>Polymer</u>	<u>Amine content (mole %)</u>
Alginate(56)	3.7
Alginate(56) - control	0.09
Alginate-tyrosinamide	4.5

When a sample of alginate-tyrosinamide-NH₂ was analyzed by SE-HPLC, it eluted at approximately the same retention time as the non-modified alginate, indicating that there was no cross-linking or degradation.

Synthesis of N-cis-aconityl-Dnm was confirmed by T.L.C. ($R_{f\text{Dnm}} = 0.09$, $R_{f\text{cisaconityl-Dnm}} = 0.3$). The amount of daunomycin bound to the alginate is shown below.

<u>Polymer</u>	<u>Dnm (% w/w)</u>
Alginate-tyrosinamide-Dnm	2
Alginate(56)-Dnm	1

Free daunomycin was not detected by T.L.C. in either preparation ($R_{f\text{Alginate-Dnm}}=0$). The daunomycin content of both polymers is low and only a small fraction of the free amine groups on the alginates reacted with the N-cis-aconityl-Dnm. This is likely to be at least in part due to steric hindrance. A higher drug content may be achieved by preparing an alginate with a higher amount of amine groups (>10 mol %), which can then be coupled

to N-cis-aconityl-Dnm. If needed, alginates of lower molecular weight can be used.

Example 2

Cytotoxicity of Alginate-Dnm conjugates

5 The effect of alginate, alginate-Dnm conjugates, daunomycin and poly-L-lysine on the viability of the mouse melanoma cell line B16 F10 *in vitro* was assessed by the MTT assay. Unmodified alginates were not toxic even at concentrations as high as 5 mg/ml. In comparison the polycation poly-L-lysine
10 was toxic at 50 mg/ml. Alginate-Dnm conjugates showed cytotoxic activity indicating that the drug activity was preserved throughout the conjugation reaction. The conjugates were approximately 500 fold less toxic than the free drug. This is expected since cellular entry of the conjugates is restricted to
15 endocytosis, a slower process than the mechanism of entry of the free drug (diffusion). Furthermore, the toxicity observed *in vitro* was mainly due to the free drug in the preparation. This was estimated by RP-HPLC and was found to be 0.61% by weight of the total daunomycin bound to the alginates.

20

Example 3

Controlled release of daunomycin from alginate-Dnm conjugates

25 Samples of alginate(56)-Dnm conjugates were incubated at 37°C in citrate/phosphate buffers at pH 5, 6 and 7 and the release of Dnm was followed by RP-HPLC analysis. The results showed that there was little Dnm released over 48 hours at pH 7. The rate of release increased as the pH decreased. At 48 hours
30 approximately 8% (5.95 mg) and 23% (15.79 mg) of the total daunomycin was released at pH 6 and 5 respectively. Figure 1 shows percent release profile, over 48 hours, of daunomycin from

the alginate-daunomycin conjugate under various conditions. Figure 2 shows $\mu\text{g/ml}$ release profile, over 48 hours, of daunomycin from the alginate-daunomycin conjugate under various conditions.

5

Example 4

In vivo antitumor activity of alginate-cis-aconityl daunomycin

Male C57BL/6J mice were injected subcutaneously with 10^5 viable B16f10 melanoma cells. Tumors were allowed to establish (approximate area 70 mm^2) and treated with a dosage of 5mg each of free daunomycin, alginate-cis-aconityl-Dnm or a mixture of alginate and daunomycin per kg mouse, using a single intraperitoneal injection. Tumor growth and survival time was monitored.

15

Alginate-cis-aconityl-Dnm caused a significant decrease in the rate of tumor growth. Figure 3 shows tumor area growth over several days.

Alginate-cis-aconityl-Dnm caused an increase in life span (14.1%). Free daunomycin increased life span by 6.25% and a mixture of alginate and daunomycin increased life span by 4.25%. Some animals receiving free daunomycin showed signs of toxicity. None of the animals receiving alginate-cis-aconityl-Dnm showed signs of toxicity. It is possible, therefore, to administer larger conjugate doses than exemplified in the Example.

20

25

30

What is claimed is:

1. An alginate-bioactive agent conjugate which
comprises:

5

(a) an alginate or alginate derivative, and

(b) a bioactive agent,

10

wherein the alginate and the bioactive agent are connected via a biodegradable spacer linkage.

15

2. A conjugate of claim 1 wherein the alginate has a molecular weight between about 2,000 and 1,000,000.

3. A conjugate of claim 2 wherein the alginate has a molecular weight between about 5,000 and 100,000.

20

4. A conjugate of claim 2 wherein the alginate has a molecular weight between about 200,000 and 1,000,000.

5. A conjugate of claim 3 wherein the alginate has a molecular weight between about 65,000 and 75,000.

25

6. A conjugate of claim 4 wherein the alginate has a molecular weight between about 600,000 and 800,000.

30

7. A conjugate of claim 1 wherein the alginate has attached functional amino groups.

8. A conjugate of claim 1 wherein the bioactive agent is a cytotoxic drug.

9. A conjugate of claim 8 wherein the cytotoxic drug is an anthracycline.

5 10. A conjugate of claim 9 wherein the anthracycline is daunorubicin, doxorubicin, epirubicin or derivatives thereof.

11. A conjugate of claim 10 wherein the anthracycline is doxorubicin.

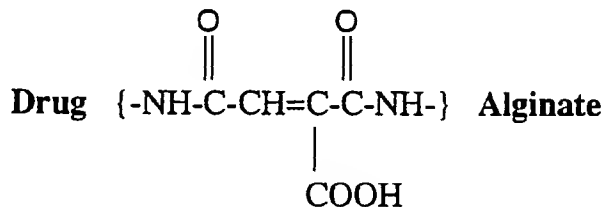
12. A conjugate of claim 1 wherein the bioactive agent is an antibacterial agent.

13. A conjugate of claim 12 wherein the antibacterial agent is amoxycillin or ampicillin.

14. A conjugate of claim 1 wherein the biodegradable spacer linkage is an acid labile linkage that is physiologically stable at vascular pH of about 7.4.

15. A conjugate of claim 14 wherein the acid labile linkage hydrolytically cleaves under acidic condition.

16. A conjugate of claim 15 wherein the acid labile linkage between alginate and drug is a cis-aconityl group having the formula



5 17. A conjugate of claim 16 wherein the alginate has attached functional amino groups and the cis-aconityl group is attached to the alginate via peptide bond.

10 18. A method for releasing a cytotoxic drug in an acidic environment comprising

15 (a) introducing an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and the cytotoxic drug are connected via an acid labile linkage, to a pH environment of about 7.4;

20 (b) modifying the pH environment of the conjugate by decreasing the pH; and

 (c) hydrolyzing the alginate-cytotoxic drug conjugate linkage.

25 19. A method for releasing a cytotoxic drug in an acidic environment comprising

 (a) introducing an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and the cytotoxic drug are connected via an acid labile linkage, into an acidic body compartment; and

30 (b) hydrolyzing the alginate-cytotoxic drug conjugate linkage.

20. A method of claim 19 wherein the acidic body compartment is a stomach or a tumor cell.

5 21. A method for delivering a cytotoxic drug to a tumor which comprises

10 (a) introducing to the tumor cell surface an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and the cytotoxic drug are connected via an acid labile linkage; and,

15 (b) hydrolyzing the alginate-cytotoxic drug conjugate linkage in the low pH surroundings of the tumor surface to release the cytotoxic drug to the tumor surroundings and absorbing the cytotoxic drug through the cellular membrane.

20 22. A method for treating a neoplastic condition in a patient having a tumor which comprises

25 (a) introducing an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and the cytotoxic drug are connected via an acid labile linkage, to a patient having a neoplastic condition; and

30 (b) hydrolyzing the alginate-cytotoxic drug conjugate linkage in the low pH surroundings of the tumor surface to release the cytotoxic drug to the tumor surroundings and absorbing the cytotoxic drug through the cellular membrane.

23. A method for delivering a cytotoxic drug to a tumor which comprises

- 5 (a) introducing an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and the cytotoxic drug are connected via an acid labile linkage, to the tumor cell surface;
- 10 (b) internalizing the conjugate by endocytosis into the tumor; and,
- 15 (c) hydrolyzing the alginate-cytotoxic drug conjugate linkage in the low pH environment of the tumor interior to release the cytotoxic drug inside the tumor.

24. A method for treating a neoplastic condition in a patient having a tumor which comprises

- 20 (a) introducing an alginate-cytotoxic drug conjugate comprising (i) an alginate or alginate derivative, and (ii) a cytotoxic drug, wherein the alginate and the cytotoxic drug are connected via an acid labile linkage, to the tumor cell surface;
- 25 (b) internalizing the conjugate by endocytosis into the tumor; and,
- 30 (c) hydrolyzing the alginate-cytotoxic drug conjugate linkage in the low pH environment of the tumor interior to release the cytotoxin inside the tumor.

25. A pharmaceutical composition comprising a therapeutically-effective non-toxic amount of at least one alginate-bioactive

agent conjugate as claimed in any one of Claims 1 to 17 together with an inert, physiologically acceptable carrier.

5 26. A medical preparation containing a therapeutically-effective non-toxic amount of a compound as claimed in any one of Claims 1 to 17 and a pharmaceutical excipient.

10 27. Use of a compound as claimed in any one of Claims 1 to 17 for the manufacture of a medical preparation for the treatment of a neoplastic condition or a bacterial infection in mammals.

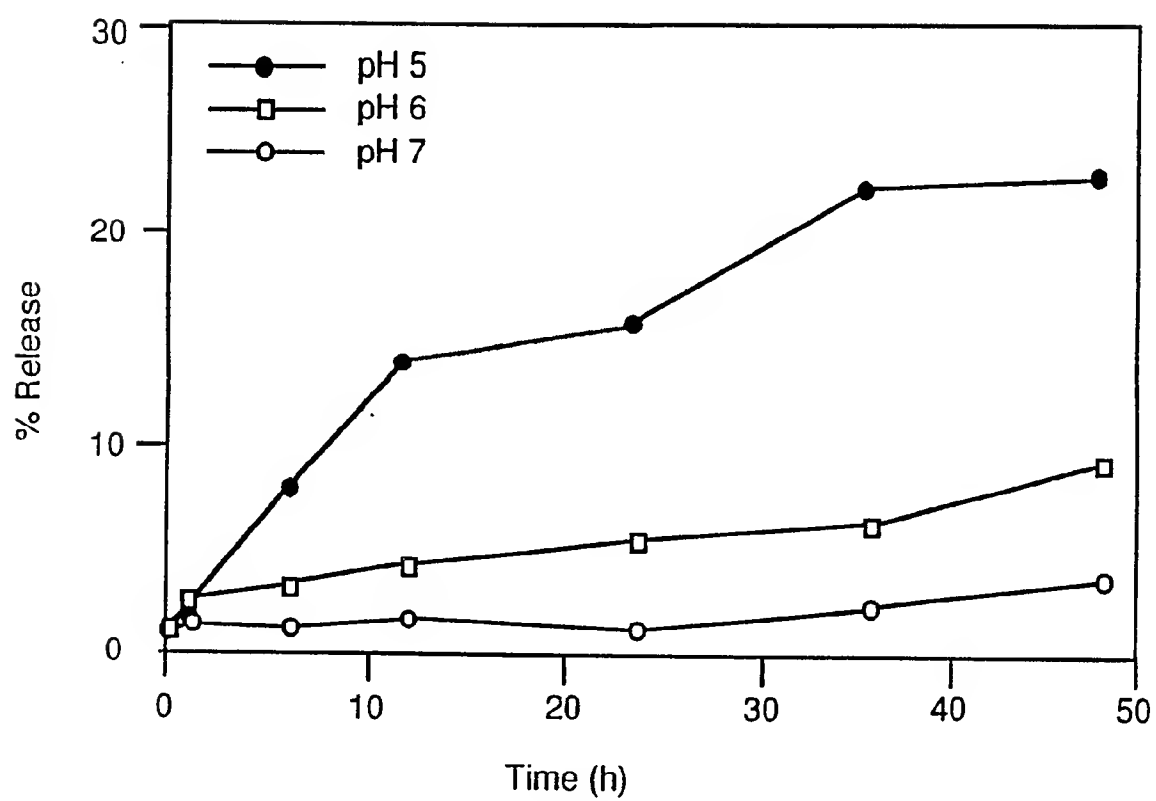
15 28. A process for preparing an alginate-bioactive agent conjugate as claimed in any one of Claims 1 to 17 from an alginate within a selected molecular weight range, a linker compound for forming the biodegradable spacer linkage, and the bioactive agent, said method comprising the steps of:

- (a) combining the linker with the bioactive agent to form a linker-modified bioactive agent;
- 20 (b) activating the alginate with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide;
- (c) combining activated alginate with ethylenediamine to form alginates having free primary amine groups (alginate-NH₂);
- (d) activating the linker-modified bioactive agent with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; and
- 25 (e) combining such activated linker-modified bioactive agent with said alginate-NH₂ to form the alginate bioactive agent conjugate.

30 29. A process as claimed in Claim 28 wherein the bioactive agent is a cytotoxic drug.

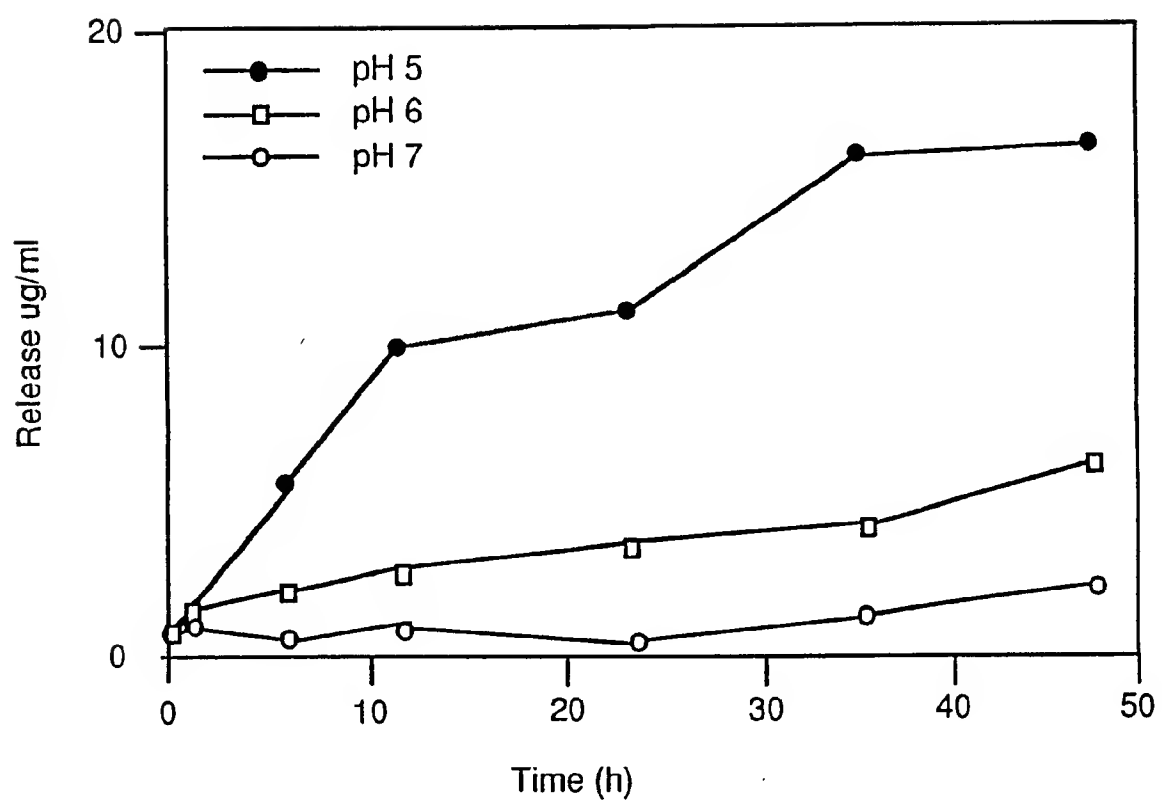
1/3

FIG. 1



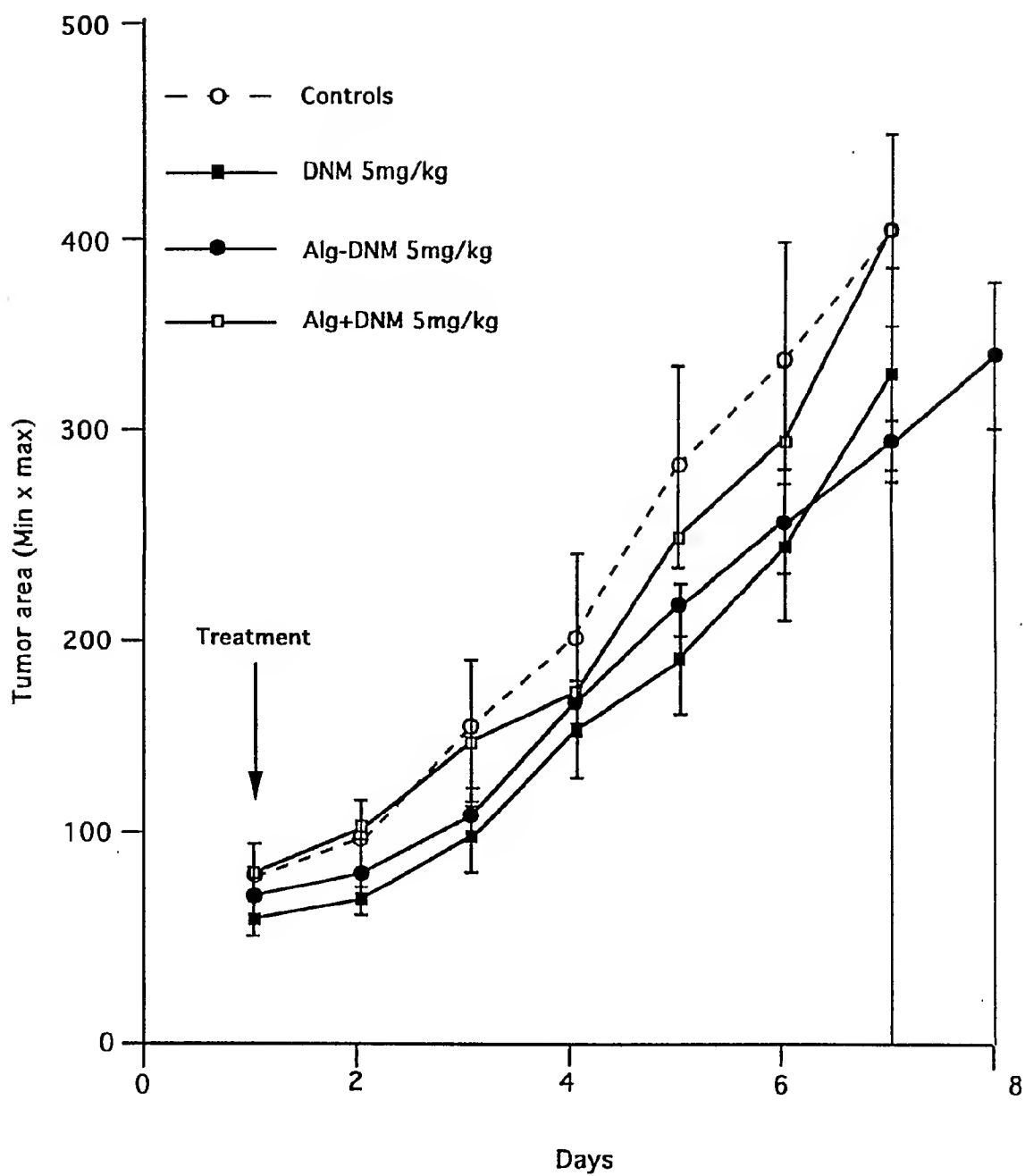
2/3

FIG. 2



3/3

FIG. 3





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 47/48	A3	(11) International Publication Number: WO 94/07536 (43) International Publication Date: 14 April 1994 (14.04.94)
(21) International Application Number: PCT/GB93/01999 (22) International Filing Date: 23 September 1993 (23.09.93) (30) Priority data: 9220294.4 25 September 1992 (25.09.92) GB (71) Applicant (for all designated States except US): KEELE UNIVERSITY [GB/GB]; Staffordshire ST5 5BG (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): AL-SHAMKHANI, Ay-men [IQ/GB]; Sir William Dunn School of Pathology, Oxford University, South Parks Road, Oxford OX1 3RE (GB). DUNCAN, Ruth [GB/IT]; Farmitalia Caro Erba, Via Carlo Imbinati, 24, I-20159 Milano (IT).		(74) Agents: SKERRETT, H., N. et al.; Charles House, 148/9 Great Charles Street, Birmingham B3 3HT (GB). (81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 21 July 1994 (21.07.94)
(54) Title: ALGINATE-BIOACTIVE AGENT CONJUGATES (57) Abstract The invention is an alginate-bioactive agent conjugate connected via an acid labile biodegradable spacer linkage. The conjugate is effective for delivering bioactive agents to targets existing in low-pH environments, either at the target surface or in the target interior. <div style="text-align: center;"> </div>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

INTERNATIONAL SEARCH REPORT

Inter. nal Application No

PCT/GB 93/01999

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOLOGY, VOL. 44, NO. 1, PAGE(S) 41-50, 1981. TAYLOR W A et al 'ADJUVANT AND SUPPRESSIVE EFFECTS OF GRASS CONJUVAC AND OTHER ALGINATE CONJUGATES ON IMMUNO GLOBULIN G AND IMMUNO GLOBULIN E ANTIBODY RESPONSES IN MICE.'	1-7, 14-17, 25-29
Y	see abstract	8-10
X	ANN ALLERGY, VOL. 56, NO. 5, PAGE(S) 384-391, 1986. BRUNO G et al 'A MULTICENTER TRIAL OF IMMUNOTHERAPY WITH ALGINATE - CONJUGATED GRASS POLLEN EXTRACT.'	1-7, 14-17, 25-29
Y	see abstract	8-10
	--- -/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

3 June 1994

Date of mailing of the international search report

16.06.94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Dullaart, A

INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/GB 93/01999

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALLERGY (CPH), VOL. 45, NO. 4, PAGE(S) 241-248, 1990. BOYE N P et al 'IMMUNOTHERAPY OF TREE POLLEN ALLERGY WITH A MODIFIED ALGINATE CONJUGATED BIRCH POLLEN EXTRACT COMPARED TO AN ALUMINUM ADSORBED EXTRACT.'	1-7, 14-17, 25-29
Y	see page 243 see page 247 ---	8-10
X	FEMS (FED EUR MICROBIOL SOC) MICROBIOL IMMUNOL, VOL. 76, NO. 4, PAGE(S) 185-192, 1991. COIN D et al 'A PSEUDOMONAS-AERUGINOSA ALGINATE EXOTOXIN A CONJUGATE THAT ELICITS ANTI- ALGINATE AND EXOTOXIN A-NEUTRALIZING ANTIBODIES.'	1-8, 14-17, 25-29
Y	see paragraph 3.4 see paragraph 4.3 ---	9,10
X	FORTY-FOURTH ANNUAL MEETING OF THE AMERICAN ACADEMY OF ALLERGY AND IMMUNOLOGY, ANAHEIM, CALIFORNIA, USA, MARCH 11-16, 1988.;& J ALLERGY CLIN IMMUNOL, VOL. 81, NO. 1, PAGE(S) 262, 1988. ORTOLANI C et al 'EFFICACY OF ONE YEAR OF SPECIFIC IMMUNOTHERAPY SIT WITH AN ALGINATE CONJUGATED PARIETARIA-OFFICINALIS EXTRACT IN PARIETARIA ALLERGIC RHINITIS.'	1-7, 14-17, 25-29
Y	* abstract 378 * ---	8-10
X	FOLIA ALLERGOL IMMUNOL CLIN, VOL. 37, NO. 3, PAGE(S) 135-142, 1990. PASTORELO E A et al 'EFFECTS OF IMMUNOTHERAPY ON IMMEDIATE AND LATE SPECIFIC NASAL REACTIVITY.'	1-7, 14-17, 25-29
Y	see abstract see page 140 - page 141 ---	8-10
X	HOIBY, N. AND S. S. PEDERSEN (ED.). INTERNATIONAL CONGRESS SERIES, NO., VOL. 977. CYSTIC FIBROSIS, BASIC AND CLINICAL RESEARCH;17TH ANNUAL MEETING OF THE EUROPEAN WORKING GROUP ON CYSTIC FIBROSIS, COPENHAGEN, DENMARK, JUNE 17-21, 1991. pages 159 - 163 CRYZ S J JR et al 'PHASE I CLINICAL EVALUATION OF A PSEUDOMONAS-AERUGINOSA ALGINATE -TOXIN A CONJUGATE VACCINE.'	1-8, 14-17, 25-29
Y	see page 159 ---	9,10

	-/--	

1

INTERNATIONAL SEARCH REPORT

Inter. Application No.

PCT/GB 93/01999

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>FORTY-SECOND ANNUAL MEETING OF THE AMERICAN ACADEMY OF ALLERGY AND IMMUNOLOGY, NEW ORLEANS, LA., USA, MAR. 21-26, 1986.; & J ALLERGY CLIN IMMUNOL, VOL. 77, NO. 1 PART 2, PAGE(S) 138, 1986. PASTORELLO E et al 'A DOUBLE-BLIND PLACEBO CONTROLLED STUDY OF SPECIFIC IMMUNOTHERAPY WITH ALGINATE CONJUGATED MITE ALPHA FRACTION IN PERENNIAL ALLERGIC RHINITIS.'</p> <p>* abstract 72 *</p>	<p>1-7, 14-17, 25-29</p>
Y	<p>---</p>	8-10
X	<p>US,A,4 003 792 (P.J. MILL ET AL.) 18 January 1977</p>	<p>1-8, 14-17, 25-29</p>
Y	<p>see claims 9-19; examples</p> <p>---</p>	9,10
X	<p>US,A,5 013 669 (D.F.PETERS ET AL.) 7 May 1991</p> <p>see the whole document</p> <p>---</p>	<p>1-7, 14-17, 25-29</p>
Y	<p>BIOCHEM. BIOPHYS. RES. COMMUN., 102(3), 1048-54 1981</p> <p>Shen, Wei Chiang et al 'cis-Aconityl spacer between daunomycin and macromolecular carriers: a model of pH-sensitive linkage releasing drug from a lysosomotropic conjugate'</p> <p>see the whole document</p> <p>---</p>	<p>7-10, 25-29</p>
Y	<p>BIOCONJUGATE CHEM, VOL. 3, NO. 2, PAGE(S) 154-159, 1992.</p> <p>MANN J S et al 'MOLECULAR AMPLIFIERS SYNTHESIS AND FUNCTIONALIZATION OF A POLYAMINOPROPYLDextran BEARING A UNIQUELY REACTIVE TERMINUS FOR UNIVALENT ATTACHMENT TO BIOMOLECULES.'</p> <p>see page 157</p> <p>---</p>	<p>7-10, 25-29</p>
Y	<p>US,A,4 631 190 (W.C. SHEN ET AL.) 23 December 1986</p> <p>see column 3 - column 7, line 31; claims; examples</p> <p>---</p>	<p>7-10, 25-29</p>
Y	<p>CANCER IMMUNOL IMMUNOTHER, 1991, VOL. 33, NO. 4, PAGE(S) 223-30,</p> <p>Lavie E et al 'Monoclonal antibody L6-daunomycin conjugates constructed to release free drug at the lower pH of tumor tissue.'</p> <p>* materials and methods *</p> <p>---</p>	<p>7-10, 25-29</p>
	<p>---</p> <p>-/--</p>	

INTERNATIONAL SEARCH REPORT

Inter. nal Application No

PCT/GB 93/01999

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE (USA), 1986, VOL. 231, NO. 4734, PAGE(S) 148-150, Diener E. et al 'Specific immunosuppression by immunotoxins containing daunomycin' see abstract ---	7-10, 25-29
Y	ANTICANCER RES, VOL. 9, NO. 4, PAGE(S) 1201-1206, 1989. GAUREAULT R C et al 'ERYTHROCYTE MEMBRANE-BOUND DAUNORUBICIN AS A DELIVERY SYSTEM IN ANTICANCER TREATMENT.' * materials and methods * ---	7-10, 25-29
Y	CANCER RES, VOL. 48, NO. 21, PAGE(S) 6097-6102, 1988. DILLMAN R O et al 'SUPERIORITY OF AN ACID-LABILE DAUNORUBICIN-MONOCLOAL ANTIBODY IMMUNOCONJUGATE COMPARED TO FREE DRUG.' * materials and methods * ---	7-10, 25-29
Y	BIANCHI, C., ET AL. (ED.). CONTRIBUTIONS TO NEPHROLOGY, VOL., VOL. 101. KIDNEY, PROTEINS AND DRUGS: AN UPDATE;7TH INTERNATIONAL SYMPOSIUM OF NEPHROLOGY, MONTECATINI TERME, ITALY, OCTOBER 14-16, 1991, S. KARGER AG: BASEL, CH pages 99 - 103 FRANSSEN E J F et al 'LOW-MOLECULAR-WEIGHT PROTEINS AS CARRIERS FOR RENAL DRUG TARGETING.' * materials and methods * -----	7-10, 25-29

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB93/01999

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

For further information please see Form PCT/ISA/206 sent 17.03.94

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 93/01999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4003792	18-01-77	BE-A- 717689 CH-A- 526590 DE-A,B,C 1768841 FR-M- 7784 FR-A- 1584763 GB-A- 1174854 LU-A- 56423 NL-A- 6809538 SE-B- 383100 SE-B- 400652 SE-A- 7511288	16-12-68 15-08-72 18-11-71 23-03-70 02-01-70 17-12-69 21-10-68 09-01-69 01-03-76 03-04-78 08-10-75
US-A-5013669	07-05-91	NONE	
US-A-4631190	23-12-86	US-A- 5144011	01-09-92